*IN VITRO SYNTHESIS OF DIFFERENT CATEGORIES OF SPECIFIC PROTEIN BY MEMBRANE-BOUND AND FREE RIBOSOMES**

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Abstract.—The free and membrane-bound ribosomes of hepatic cells were isolated and used to program protein synthesis in cell-free extracts. Immunoelectrophoretic and radioautographic analysis of the products showed that the membrane-bound ribosomes synthesized serum proteins, whereas ribosomes free of endoplasmic reticulum synthesized specific nonserum liver proteins. After synthesis, the serum proteins remained associated with membrane vesicles, whereas the other liver proteins were found free of cytoplasmic organelles.

The ribosomes of secretory cells are found associated with the endoplasmic reticulum or they can be isolated apparently free of these membranes.[1–3] In 1960, Siekevitz and Palade[4] observed that most of the newly synthesized pancreatic α-chymotrypsinogen appeared associated with ribosomes attached to membranes. They suggested that membrane-bound ribosomes are engaged in the synthesis of exportable proteins, whereas free ribosomes synthesize primarily nonexportable proteins. Similarly, serum albumin labeled in hepatic cells is found attached to membrane-bound ribosomes.[5–11]

Work from this and other laboratories[5, 9–11] has established that serum proteins can be synthesized in rat or mouse liver cell-free extracts and that the newly finished serum proteins remain associated with the microsomal vesicles. Other labeled proteins appear free of these organelles, however, and we have reported[12] that those specifically detected are almost exclusively nonserum proteins. In this communication we show that, in cell-free extracts, serum proteins are made on ribosomes attached to membranes and that several nonserum proteins are synthesized on free ribosomes. These results conclusively demonstrate a functional distinction between these two classes of ribosomes previously suggested from experiments *in vivo*.[4]

Materials and Methods.—Isolation of free and bound ribosomes: Rat livers were homogenized as previously described[10] in 0.25 *M* sucrose in TKM buffer (0.01 *M* $MgCl_2$, 0.025 *M* KCl, 0.01 *M* Tris (HCl) pH 7.8). Homogenates were centrifuged 10 min at 30,000 *g*, and 4 ml of the resulting supernatant fraction were layered on discontinuous sucrose density gradients[13] containing 3 ml of 2 *M* sucrose in TKM buffer and 3 ml of 0.5 *M* sucrose in TKM buffer. These were centrifuged for 7 hr at 150,000 *g*. Free ribosomes were recovered in the pellet, and bound ribosomes remained on the 2 *M* sucrose shelf.

Electron micrographic examination of free ribosomes, a courtesy of David Sabatini, revealed a small contamination of free ribosomes by rough membranes. Phospholipid could not be detected in free ribosomes, however, an indication of less than 10% contamination by bound ribosomes. The extent of contamination of the bound ribosome preparation with free ribosomes was not assessed.

Incubation mixtures: Incubation mixtures for cell-free protein synthesis contained 27 mM Tris (HCl) buffer, pH 7.8, 0.5 mM GTP, 8 mM GSH, 2 mM ATP, 8 mM PEP, 6

mM $MgCl_2$, 50 mM KCl, and 0.06 mM each of tryptophan, methionine, asparagine, glutamine, histidine, cysteine, and glycine. In addition, each milliliter of the incubation mixture contained 40 μg pyruvate kinase, 2.5 mg RNA equivalents of ribosomes (either free or bound), 0.01 mg liver tRNA, and 2.5–6.0 mg protein of dialyzed 105,000 *g* supernatant fraction of the liver homogenate. Four μCi of a labeling ^{14}C mixture containing the other thirteen amino acids ("Reconstituted protein hydrolysate," Schwarz Bio-Research, Inc.) were added to each milliliter of incubation mixture. Incubation was at 37°C for 50 min.

Fractionation of the incubation mixtures: Labeled proteins were recovered in the supernatant fraction after centrifugation at 105,000 *g* for 90 min. The ribosomes or the microsomal pellets were suspended in 0.15 *M* NaCl and sonicated to liberate any sequestered products. Ribosomes and membranes were sedimented again at 105,000 *g* for 2 hr. Products in all supernatant fractions were concentrated by precipitation with ammonium sulfate to 80% saturation and were dialyzed against 0.038 *M* barbital buffer, pH 8.2, prior to immunoelectrophoresis and autoradiography.

Antiserum: Antibodies against soluble rat liver antigens were prepared in rabbits by one intraperitoneal, two intramuscular, and two subcutaneous injections of antigen solution emulsified in complete Freund's adjuvant (Difco), 0.5 ml/site. After 2 months, biweekly series of two intravenous injections of aqueous antigen (10 mg protein/ml) were begun and continued until a sufficient number of antibodies for distinct antigens were present in the serum. Sera from two rabbits were pooled, and rat serum antigens were added until no reactions with rat serum were detectable. After removing antibodies to serum proteins, anti-rat liver antibodies were concentrated three times by salting out with Na_2SO_4 at 18%.

Antisera against rat serum antigens were prepared by a similar procedure, except that removal of nonspecific antibodies and concentration were not required.

Immunoelectrophoretic analysis and autoradiography: Microelectrophoresis in agar was performed at 6 volts per cm for 90 min in 0.038 *M* sodium barbital buffer, pH 8.2. All analyses were performed in duplicate, some in triplicate. Each labeled protein preparation from the incubation mixtures was analyzed with and without carrier proteins of the type corresponding to the antiserum. The amount of carrier was carefully matched with the antiserum to give optimal patterns for certain component antigens. Antibody diffusion was continued for 48 hr with antiserum against rat liver antigens, 24 hr with antiserum against rat serum proteins.

Washed and dried plates (microscope slides) were placed against a Kodak No-Screen emulsion on photo plates cut to 25 × 75 mm. Exposure times varied from 6 to 25 weeks.

Other methods: Incorporation of radioactive amino acids into protein and determination of protein and RNA concentrations were described in a preceding report.[10] Liver tRNA was prepared by the method of Gierer and Schramm.[14] Phospholipids were extracted by the Folch procedure.[15] Phospholipid total phosphorus was determined as described by Ames.[16]

Results.—To test the hypothesis that serum proteins are synthesized on ribosomes bound to the endoplasmic reticulum whereas nonserum proteins are made on free ribosomes, the following experiment was done. Free and bound ribosomes were isolated from rat liver by sedimentation on sucrose gradients (see *Materials and Methods*) and were used in cell-free protein synthesis. The products of synthesis were analyzed by immunoelectrophoresis and radioautography. The specific activity of labeled acid-insoluble peptides obtained with total, free, or membrane-bound ribosomes is shown in Table 1. The amount of ^{14}C -amino acids incorporated per milligram of RNA in free ribosomes was higher than that obtained with bound ribosomes. This differs from data of *in vivo* experiments where bound and free ribosomes were reported to be equally active in synthesis.[7] The discrepancy might be due to partial inactivation of bound

TABLE 1. *Incorporation of ^{14}C-amino acids into protein by free, bound, and total ribosomes in cell-free incubation mixtures.*

Experiment	dpm incorporated/mg RNA		
	Total	Free	Bound
1	126,500	130,250	42,500
2	117,900	109,900	50,500

Mixtures were incubated for 50 min at 37°C.

ribosomes during isolation. For experiment 2 a small amount of soluble cell extract was added to the sucrose solutions as RNase inhibitor in the discontinuous gradients used to prepare the free and bound ribosomes.[17] This precaution did not make a significant difference in the activity of these fractions, but a larger amount of specific liver proteins were labeled in experiment 2 than in experiment 1.

Approximately half the total acid-insoluble protein synthesized by free ribosomes was released into the supernatant fraction of the incubation mixture. This contrasts with a much lower 5 per cent released by the bound ribosomes. Sonication of the bound ribosomes after incubation freed an additional 10 per cent of the total labeled proteins, while only about 2 per cent more label was released from the free ribosomes by sonication.

Protein freed by sonication of ribosomal fractions or protein found free in the first high-speed supernatant fraction was concentrated with ammonium sulfate and analyzed as described in *Materials and Methods*.

Figure 1 shows that the serum proteins, albumin and transferrin, are synthesized by bound ribosomes. Neither of these proteins was detected in the first high-speed supernatant fraction. Serum proteins normally remained associated with the endoplasmic reticulum and disruption of the vesicles was required to recover them. The pattern shown in Figure 1 was prepared with nonradioactive serum proteins as carrier, diluted to place albumin and transferrin in equivalence with their respective antibodies. (More efficient autoradiograms are obtained with equivalence reactions.) When less dilute carrier is used, several less-prominent rat serum α and β globulins are in equivalence, and the corresponding autoradiograms show four or five of them to be labeled, with albumin and transferrin precipitates partially dissolved in antigen excess. Albumin and transferrin shown in Figure 1 are therefore taken as type components representing a larger group. Antiserum against liver proteins, as we show in a more complete report,[12] reveals no labeled liver components released by sonication of the ribosome-membrane complex. The first high-speed supernatant fraction of the bound ribosome incubation contained very faint traces of nonserum proteins. This may have been due to contamination by free ribosomes.

Analysis of the supernatant products of the free ribosome incubation mixture is shown in Figure 2. Only the immunoelectrophoretic analysis patterns of nonserum liver proteins are shown, since we detected no labeled serum proteins in this preparation. The several distinct radioactive bands visible in the autoradiogram in Figure 2 are therefore nonserum liver components labeled and released by free ribosomes. No detectable labeled liver antigens can be released by sonication of the ribosomes even though a small amount of labeled peptide is freed by

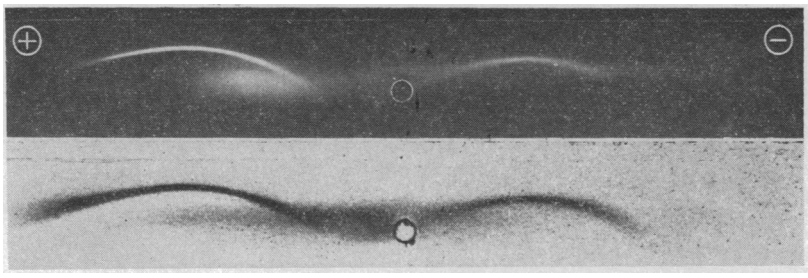

FIG. 1.—Labeling of serum proteins by bound ribosomes. *Top*: Immunoelectrophoretic analysis pattern of 10-times-diluted rat-serum carrier (2.5-μl) supplemented with concentrated labeled proteins (16 μg) liberated from bound ribosomes (microsomal vesicles) by sonication after *in vitro* incubation. Pattern was developed by rabbit antiserum against rat-serum proteins. The albumin reaction is that nearest the anode (*left*); the reaction of the slower transferrin is to the right.

Bottom: Autoradiogram of above pattern. At this carrier dilution only albumin (*left*) and transferrin (*right*) are near antigen-antibody equivalence; thus, only these components are sharply revealed.

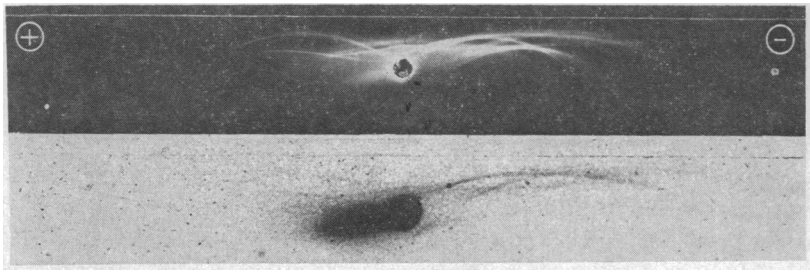

FIG. 2.—Labeling of nonserum proteins by free ribosomes. *Top:* Immunoelectrophoretic analysis pattern of proteins (12 μg) concentrated from the 105,000 *g* supernatant fraction of the *in vitro* incubation mixture prepared with free ribosomes. The pattern was developed by rabbit antiserum against rat liver extract. Antibodies against serum proteins had been previously removed.

Bottom: Autoradiogram of above pattern. At least seven distinct nonserum components were labeled by free ribosomes *in vitro*.

such treatment. There were traces of labeled albumin released by sonication of the ribosomes, however, and this is demonstrated in Figure 3. This finding is consistent with the presence of a small microsomal contamination in the free ribosome preparation as reported in *Materials and Methods*. The relative significance of the active contaminant is suggested by comparing the autoradiogram of Figure 3 with that of Figure 1.

Discussion.—The results of these experiments demonstrate that several serum proteins are made on ribosomes bound to the endoplasmic reticulum and that several nonserum soluble proteins of hepatic cell extracts are made on free ribosomes. Even though the preparations of free and bound ribosomes may have been slightly contaminated, each with the other, a large enough number of immunologically distinct proteins of each class were synthesized to suggest that the partitioning of these ribosomes has special functional significance. Hicks *et al.*[18]

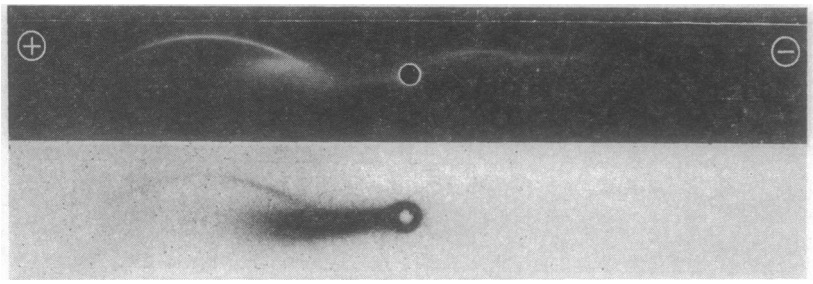

FIG. 3.—Labeling of serum albumin in free ribosome preparation presumably by contaminant bound ribosomes. *Top:* Immunoelectrophoretic analysis pattern of 20-times-diluted rat-serum carrier (2.5 μl) supplemented with concentrated material liberated by sonication from the free ribosome preparation after incubation and sedimentation. Pattern was developed by rabbit antiserum against rat serum proteins.

Bottom: Autoradiogram of above pattern showing faint labeling of albumin. Compare with labeling of albumin in Fig. 1.

have recently reported that ferritin, a nonexported protein, is preferentially labeled by free ribosomes.‡ Redman[19] reports similar findings.

Not all the immunologically identifiable serum proteins which are made in the liver[20, 21] were detectably labeled by *in vitro* systems. Similarly, only a few of the many protein antigens revealed in the liver extracts were detectably labeled (Fig. 2). Three explanations readily come to mind: (1) Some proteins are synthesized very slowly so that an insufficient quantity is labeled during the 50-minute incubation *in vitro;* (2) some finished chains may not be assembled into multichain proteins *in vitro* and therefore may not be identified with the native protein of the carrier; (3) certain proteins may be made on some other population of the ribosomes.

The serum proteins synthesized in these cell-free extracts or *in vivo* are not released from the endoplasmic reticulum into the supernatant fraction unless the membrane vesicles are disrupted by sonication or by other means.[5–11] Similarly, puromycin peptides synthesized on microsomes remain entrapped within the lumen of the microsomal vesicles.[22] In contrast, the nonserum proteins synthesized on free ribosomes were found free of membrane components. All these observations are consistent with the hypothesis that ribosomes are oriented on the endoplasmic reticulum in a manner which allows the unidirectional extrusion of protein destined for export.[4, 23]

These experiments do not exclude the possibility that some nonexported proteins are synthesized on the bound ribosomes. Over 80 per cent of the peptides synthesized by bound ribosomes remain tightly associated with the pellet, while only 50 per cent remain associated with the free ribosomes after sonication. It is probable that the latter figure represents nascent protein esterified to tRNA and that a similar proportion would hold for the bound ribosomes. It is possible, therefore, that the balance represents incorporation of label into membrane proteins which remain part of the microsome structure. There is evidence that some membrane proteins are made on bound ribosomes,[24] though microsomal NADPH cytochrome *c* reductase is apparently synthesized on both free and bound

ribosomes.[25] It is known that certain cells which do not seem to export protein do have ribosomes attached to the endoplasmic reticulum.[26] Our methods would not identify membrane proteins, however, unless they were released in a soluble form into the supernatant fraction of the incubation mixture or into the lumen of the microsomal vesicle. If membrane proteins are made on bound ribosomes identical to those which produce exportable proteins, it would be expected that the discharge of these would also be vectorial across the membrane into the lumen of the microsome, a mechanism proposed by Redman and Sabatini.[22] If they are thus discharged, all that can be said is that they are not soluble under our extraction conditions or that we have no specific antibodies for them.

These considerations notwithstanding, we find that a group of specific nonserum liver proteins are made on free ribosomes and not appreciably, if at all, on ribosomes bound to the endoplasmic reticulum. We can conclude, therefore, that hepatic-free ribosomes are functional entities and are not degradation products artificially detached from the endoplasmic reticulum.

The biochemical mechanism by which the synthesis of unique proteins is partitioned between free and bound ribosomes is unknown. Specifically, it is not known whether the association of ribosome and membrane is the consequence of the synthesis of particular proteins or whether the corresponding messengers seek out ribosome-membrane complexes prior to peptide chain initiation. It is known, however, that the 60*S* subunit bearing peptidyl-tRNA is bound to the endoplasmic reticulum,[27] while the 40*S* particle which binds messenger RNA is not. It is likely, therefore, that the large particle or the nascent chain on this subunit recognizes the membrane.

There is evidence that peptide-chain initiation in bacteria[28–31] and in certain mammalian cells[32–34] requires that the ribosomal subunits be unjoined. If this apparently general phenomenon holds true for both free and bound ribosomes in hepatic cells, and if the 60*S* subunits do differ in their specificity for membranes, then the 40*S* particle-messenger complex must have additional recognition mechanisms for the bound 60*S* particle. There are few data on ribosomal differences, however, and reports on rates of RNA synthesis in separate ribosomal compartments[35, 36] are conflicting.

If the sequence of events in chain initiation is accepted and if the 60*S* particles of bound and free ribosomes do not differ in some significant way, attachment to the membrane must occur after peptide synthesis has begun. The segregation mechanism is, therefore, likely to be messenger-directed and in part a function of the nascent peptide chain.

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‡ In August 1968, we communicated the above findings to Dr. Munro and at his request sent him a brief written summary for citation in his recent report with Drs. Hicks and Drysdale.[18] The paragraph was inadvertently deleted in the revised and published copy. Dr. Munro has kindly submitted a correction which will be incorporated into reprints.

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